Arch Toxicol (1998) 72: 499–504

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Biomonitoring of aromatic amines V: acetylation and deacetylation in the metabolic activation of aromatic amines as determined by haemoglobin binding

Received: 19 January 1998 / Accepted: 7 April 1998

Abstract Aromatic amines are metabolically activated by N-oxidation of either the amine or the acetamide as a first step and esterification of the resulting N-hydroxyl derivatives as a second step. Both pathways may lead to DNA-adducts and subsequently to DNA lesions and mutations. Since the accumulation of non-acetylated adducts has been associated with tumour initiating properties, the balance between acetylation and deacetylation may greatly influence the biological effect. Hydrolysable haemoglobin adducts representing the bioavailability of N-hydroxylamines and the corresponding nitroso-derivatives were analysed following oral administration to female Wistar rats of two arylamine-acetamide couples: 4-aminobiphenyl and 2-aminofluorene, and two arylamine-acetamide-diacetamide triples: benzidine and 3,3′-dichlorobenzidine. The results show that the monoacetamides are readily deacetylated in vivo whereas the diacetamides are not. A dynamic equilibrium is indicated to exist between acetylation and deacetylation, which depends on substrate specificity, and the role of deacetylation is emphasised. In addition, acetylation polymorphism was studied with 4-chloroaniline and 3,3′-dichlorobenzidine in slow acetylating A/J and rapid acetylating C57BL/6J mice. The slow acetylator genotype was associated with significantly higher haemoglobin-adduct levels for both arylamines. The results provide additional support for the use of haemoglobin adducts in biomonitoring as a dosimeter for the biologically active dose of arylamines/arylacetamides. Moreover, biomonitoring of haemoglobin adducts may provide information about an individual's susceptibility to the toxic and carcinogenic effects of these chemicals.

Abbreviations *AB* 4-Aminobiphenyl · *AF* 2-aminofluorene · *AAF* 2-acetylaminofluorene · *BZ* benzidine · *DCB* 3,3′-dichlorobenzidine · *HBI* haemoglobin binding index

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Key words Biomonitoring · Haemoglobin adducts · Aromatic amines · Acetylation · Deacetylation

Introduction

The biologically active dose of acutely toxic and carcinogenic aromatic amines depends on the bioavailability of N-oxidation products, which is greatly influenced by the balance between acetylation and deacetylation (Beland and Kadlubar 1990). N-Acetylation is often considered as inactivation or detoxification step because it lowers the concentration of the primary amine, the substrate for N-oxidation to the proximate genotoxin N-hydroxylamine. The N-hydroxylamine is readily oxidised to the nitroso-derivative, which reacts with SH-groups of cysteine and yields stable protein adducts, particularly with haemoglobin in erythrocytes (Dölle et al. 1980; Eyer 1994). In previous reports we described the use of arylamine-haemoglobin (Hb) adducts as dosimeters for the bioavailability of metabolically formed N-oxidation products (Birner et al. 1990; Zwirner-Baier et al. 1994). Whereas N-acetyltransferase-mediated acetylation has attracted much attention (Weber 1987; Meyer 1994), deacetylation and the interplay between the two has not (King and Weber 1981; Gant et al. 1994; Grant et al. 1997).

The human carcinogen benzidine seemed to be a suitable example to study this interplay. As a bifunctional amine three acetylation stages exist: the non-acetylated diamine, the monoacetamide and the diacetamide (Fig. 1). Martin et al. (1982) identified the DNA-adduct as N-(deoxyguanosin-8-yl)-N′-acetylbenzidine and proposed the N-hydroxy derivative of the N′-monoacetamide as the proximate genotoxin. Accordingly, monoacetylbenzidine has been identified as one of the cleavage products from the sulphinic acid type Hb-adducts after oral administration of benzidine to rats (Birner et al. 1990, Fig. 1). The diamine, however, was also found and from the ratio monoacetylbenzidine to benzidine of 8:1, it was concluded that monoacetyl-

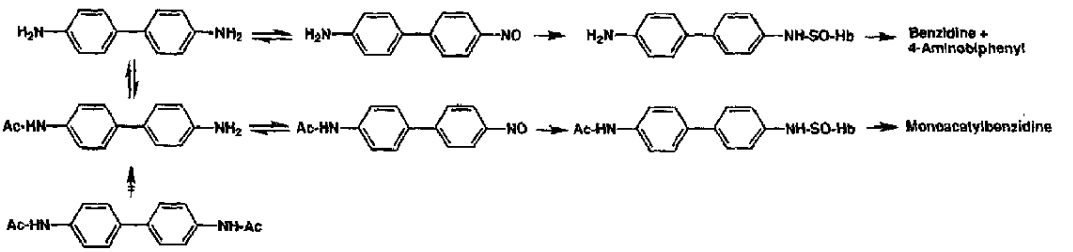

Fig. 1 Formation of hydrolysable haemoglobin adducts of the sulphinic acid amide-type in the metabolism of benzidine and the resulting cleavage products

ation was indeed a prerequisite to form the ultimate genotoxin in vivo. If this were the case administration of the precursor should give higher adduct yields. If, however, deacetylation is also involved this scenario may not apply. Moreover, the addition of benzidine diacetamide should also give Hb-adducts.

To demonstrate the role of acetylation and deacetylation, two amine-acetamide couples (4-aminobiphenyl, 2-aminofluorene) and two amine-acetamide-diacetamide triples (benzidine and 3,3′-dichlorobenzidine) were eventually administered to female Wistar rats and the Hb-adducts measured after 24 h. In addition, ^{14}C-labelled 4-chloroaniline and 3,3′-dichlorobenzidine were administered to slow acetylating A/J- and rapid acetylating C57BL/6J mice (Martell et al. 1992) and the Hb-adducts determined to study the role of the acetylator phenotype.

Materials and methods

Chemicals

Radiolabelled [^{14}C]-4-chloroaniline (3.5×10^7 Bq/mmol, purity >98%) and [^{14}C]-3,3′-dichlorobenzidine (17.8×10^7 Bq/mmol, purity >98%) were purchased from Sigma (Sigma-Aldrich, Deisenhofen, Germany). 3,3′-Dichlorobenzidine (DCB), 4-aminobiphenyl (AB), benzidine (BZ), 2-aminofluorene (AF) were purchased from Aldrich (Sigma-Aldrich) and 3,3′-dichlorobenzidine hydrochloride from Sigma. Free amine was prepared by dissolving the hydrochloride in water and adding dropwise 1 *N* NaOH; the amine was extracted from the alkaline solution with ether. The amines were acetylated with acetanhydride in our laboratory (Birner et al. 1990) and purity of all substances was checked by high-performance liquid chromatography/UV spectrophotometry (HPLC/UV) and gas chromatography/mass spectrometry (GC/MS) in the electron ionisation (EI) mode: N-acetyl-3,3′-dichlorobenzidine contained < 1 ppm DCB; N,N′-diacetyl-3,3′-dichlorobenzidine contained < 1 ppm DCB and < 1 ppm N-acetyl-3,3′-dichlorobenzidine; N-acetylaminobiphenyl contained < 1 ppm AB; N-acetylbenzidine contained < 1 ppm BZ; N,N′-diacetylbenzidine contained < 1 ppm BZ and < 1 ppm N-acetylbenzidine; and N-acetylaminofluorene contained < 1 ppm AF. If necessary, substances were recrystalized once or more.

Animals and administration

Slow (A/J) and rapid (C57BL/6J) acetylating mice, body weight 18–22 g, were obtained from Zentralinstitut für Versuchstierkunde, Hannover, Germany. Radiolabelled [^{14}C]-4-chloroaniline and [^{14}C]-3,3′-dichlorobenzidine were dissolved in propanediol/ethanol (1:1) and 100 µl administered by gavage; 0.2 mmol/kg of each compound was given to 5 female mice. Female Wistar rats (Zentralinstitut für Versuchstierkunde) of body weight 200–250 g were administered 0.2 mmol/kg of the test compounds. If possible the substances were dissolved in propanediol, otherwise in different mixtures of ethanol/1,2-propanediol (2:1 or 1:1) and 200–250 µl administered by gavage to three animals per compound. The diacetamides were given as suspensions (300–350 µl). After 24 h blood was taken by heart puncture under ether anaesthesia and haemoglobin and plasma proteins (only for mice) were isolated (Zwirner-Baier and Neumann 1994).

HPLC analysis

To determine hydrolysable haemoglobin adducts, 10–100 mg haemoglobin was dissolved in 3.6 ml 1 *N* NaOH/0.05% sodium dodecyl sulphate (SDS) for 1 h at room temperature. Before hydrolysis a recovery standard was added (using one of the other amines, i.e. after feeding 4-AB, benzidine was used). After centrifugation the mixture was absorbed onto Polysorb MP-1 columns (100 mg, Interaction Chemicals, San Jose, Calif., USA) conditioned with hexane, 2-propanol, methanol and water. The columns were extracted with 1 ml of acetonitrile. An internal standard (one of the acetamides) was added. In case of the diacetamides, the extract was concentrated under a stream of nitrogen to 50 µl to increase sensitivity. To determine recoveries, haemoglobin from control animals was spiked with the various amines and their acetamides. Recoveries were between 82 and 98%. To control for non-covalently bound amines or monoacetylamines, haemoglobin samples were suspended in 3.6 ml water/0.05% SDS. The suspension was stirred for 1 h at room temperature. Under these conditions non-covalently bound amines or acetamides could only be detected in the case of 4-chloroaniline at 1–2%.

The extracts were analysed by HPLC with UV-detection combined with electrochemical detection (ECD) using a Waters model 590 HPLC pump (Waters, Milford, Mass., USA). The UV detector (Spectra Focus; Spectra Physics, San Jose, Calif., USA) was set at 280 nm, the electrochemical detector (ESA; Coulochem II, Bedford, Mass., USA) equipped with a high sensitive analytic cell (model 5011) was set at potentials of 0.5–0.85 V. The following columns were used: precolumn 20 × 2 mm (Upchurch Scientific, GAT GmbH, Bremerhaven, Germany) filled with Perisorb RP-18, 30–40 µm (Merck, Darmstadt, Germany), two different analysing columns LiChrospher 60 RP-Select B, 5 µm, 250 × 4 mm (Merck) or Zorbax ODS, 5 µm, 250 × 4 mm (Bischoff Analysentechnik, Leonberg, Germany). The following eluants were used: BZ, N-acetyl-BZ, 4-AB, 70% sodium phosphate buffer (0.02 M, pH 5.8), 20% methanol, 10% acetonitrile; AF, 45% sodium phosphate buffer (0.02 M, pH 5.8), 55% methanol; DCB, N-acetyl-DCB, 50% sodium phosphate buffer (0.02 M, pH 5.4), 40% methanol, 10% acetonitrile; 4-chloroaniline, 50% sodium phosphate buffer, 50% methanol.

Chromatograms were evaluated by integration software from PE Nelson (Cupertino, Calif., USA) or the PC 1000 System Software from TSP-GmbH (Darmstadt, Germany). Calibration curves were established by measuring standard solutions of amines and their acetamides at five different levels (UV-detection, 2, 5, 10, 50 and 100 ng in 20 µl; ECD, 100, 500 pg, 1, 5 and 10 ng in 20 µl).

Every day a two level calibration was made. By comparison with the calibration curve a factor was calculated and the chromatograms evaluated accordingly. The detection limit for DCB, N-acetyl-DCB, BZ and N-acetyl-BZ was 6 ng/g Hb with EC-detection. Haemoglobin adduct formation is expressed as haemoglobin binding index [HBI: binding (nmol substance/mol Hb)/dose (mmol/kg body weight)].

Experiments with [^{14}C]-radiolabelled compounds were quantified by scintillation counting using Rotiscint 2000 as scintillation cocktail and a scintillation counter from Wallac (LKB, Freiburg, Germany). Protein was discolored by adding 100 µl 30% H_2O_2. Total haemoglobin binding was determined by counting the total protein precipitates, the hydrolysable fraction by counting the extract (2 × 3 ml ether) after alkaline hydrolysis, or using the same procedure as for the unlabelled amines.

Results

4-Aminobiphenyl and 2-aminofluorene

The two arylamines and their acetamides were orally administered to female Wistar rats and Hb-adducts determined after 24 h. Adducts were found in all four cases, the Hb-binding index being considerably higher with the biphenyl derivative as anticipated (Table 1). The results demonstrate that the administered acetamides were deacetylated extensively. If administered, the Hb-adduct levels were almost as high as those with the amines, to one-half and one-third of the levels, respectively.

Benzidine and 3,3′-dichlorobenzidine

The situation is more complicated with these bifunctional arylamines. With benzidine three hydrolysable Hb-adducts are formed (Table 1). A small fraction of non-acetylated benzidine is N-oxidised at one of the two amino-groups to an amino-hydroxylamino-derivative and further to an amino-nitroso-derivative, which reacts with the SH-group of cysteine in Hb and yields the diamine upon hydrolysis. A much greater proportion (17-fold) of benzidine is acetylated and apparently N-oxidised in its monoacetylated form. This pathway yields monoacetylbenzidine as the cleavage product. Another small fraction of benzidine is deaminated and yields 4-aminobiphenyl as the cleavage product. The results show that the biologically active dose is not much different when feeding benzidine instead of the monoacetamide. This indicates that an equilibrium exists in vivo between monoacetylated and deacetylated benzidine, which lies heavily on the acetylated side. Surprisingly, benzidine diacetamide seems not to be deacetylated at all, or the equilibrium is extremely one-sided. The result also makes it rather unlikely that N-hydroxylation of benzidine diacetamide and subsequent N,O-transacetylation (Morton et al. 1979) has contributed significantly to the metabolism of benzidine diacetamide.

With 3,3′-dichlorobenzidine much lower adduct levels are found, deamination does not take place and the equilibrium is on the side of the deacetylated derivative (Table 1). Whereas the monoacetamide is clearly deacetylated, and the adduct levels are indistinguishable from those obtained with the diamine, the diacetamide is not deacetylated at all.

The acetylation polymorphism

4-Chloroaniline

Using ^{14}C-labelled 4-chloroaniline, Hb-adduct formation is demonstrated to depend strongly on the acetylator status in this mouse model. With slow acetylating A/J-mice, total Hb-binding was considerably higher than with the rapid acetylating C57BL/6J-mice (Table 2). With the labelled chemical it was possible to differentiate between total and hydrolysable binding and also to measure binding to plasma proteins. The results showed that non-hydrolysable Hb-adducts were formed

Table 1 Role of deacetylation for the metabolic activation of N-arylamides as indicated by haemoglobin binding (*ABZ* N-Acetylbenzidine, *ADCB* N-acetyl-3,3′-dichlorobenzidine, *3,3′-DCB* 3,3′-dichlorobenzidine)

Substance administered	Cleavage products (nmol/g Hb)			Ratio	HBI (hydrolysable)
4-Aminobiphenyl	4-AB				
4-AB	2249 ± 272[a]				725 ± 88
N-Acetyl-4-AB	1379 ± 152				445 ± 49
2-Aminofluorene	2-AF				
2-AF	150 ± 50				48 ± 16
N-Acetyl-2-AF	57 ± 10				18 ± 3
Benzidine	BZ	ABZ	4-AB		
Benzidine	8.7 ± 2.7	147 ± 50	8.9 ± 6.5	1:17:1	53 ± 19
N-Acetylbenzidine	3.3 ± 1.1	98 ± 38	3.6 ± 1.8	1:30:1	34 ± 13
N,N′-Diacetylbenzidine	<0.2	<0.2	<0.2		
3,3′-Dichlorobenzidine	3,3′-DCB	ADCB			
3,3′-DCB	3.6 ± 1.2	0.7 ± 0.1		5:1	1.4 ± 0.4
N-Acetyl-3,3′-DCB	4.8 ± 1.2	1.0 ± 0.1		5:1	1.9 ± 0.4
N,N′-Diacetyl-3,3′-DCB	<0.2	<0.2			

[a] Mean ± SD, $n = 3$

Table 2 Haemoglobin binding of slow acetylating (A/J) and rapid acetylating (C57BL/6J) mice (n = 5) 24 h after oral administration of [^{14}C]-chloroaniline and [^{14}C]-3,3′-dichlorobenzidine (0.2 mmol/kg). The hydrolysable fraction was determined from pooled proteins of 5 animals

	Haemoglobin			Plasma protein	
	Total binding (nmol/g Hb)	Hydrolysable (%)	HBI (total)	Total binding (nmol/g Hb)	Hydrolysable (%)
4-Chloroaniline					
A/J	346 ± 63	74	112	71 ± 2	71[a]
C57BL/6J	16 ± 4	43	5	16 ± 4	29[a]
3,3′-Dichlorobenzidine					
A/J	56 ± 0.4	10–15	18	56 ± 0.8	21
C57BL/6J	7 ± 0.4	5–10	2	21 ± 0.8	12

[a] Determined after hydrolysis and extraction by high-performance liquid chromatography (HPLC)-analysis

and that this fraction was greater in the rapid acetylators. Moreover, the relative fraction bound to plasma proteins was higher in slow than in rapid acetylators.

3,3′-Dichlorobenzidine

With [^{14}C]-3,3′-dichlorobenzidine similar results were obtained, except that the hydrolysable fraction of Hb-adducts was much smaller and plasma protein binding was relatively higher than with 4-chloroaniline (Table 2).

Discussion

Aromatic amines are metabolically activated by two major pathways. (1) Via N-hydroxylation of N-acetylarylamines and subsequent O-esterification of the hydroxamic acid leading to N-acetylated arylamine adducts at C8 of deoxyguanosine, and (2) via N-hydroxylation of arylamines, which are either reactive as such or are further activated by O-esterification, leading to non-acetylated C8 deoxyguanosine adducts (Beland and Kadlubar 1990). In the case of AAF, the acetylated adduct distorts DNA conformation much more than the non-acetylated adduct (Daune et al. 1981). The adduct is therefore more readily recognised and repaired. The non-acetylated adduct accumulates and has been associated with the tumour initiating properties of N-hydroxy-AAF and N-hydroxy-N-acetyl-AB, whereas the acetylated adducts block DNA replication and are correlated with the clastogenic properties of the compounds (Meerman and van de Poll 1994). The preparation of specific antibodies greatly facilitated the measurements of the two types of adducts (Poirier and Yuspa 1981) and these authors have already emphasised the role of deacetylation. Kriek (1974) originally found the deacetylated C8 adduct to represent the largest fraction (70%) in rat liver after injection of N-hydroxy-AAF or N-acetoxy-AAF. Lai et al (1988) considered deacetylation of N-hydroxy-AAF to N-hydroxy-AF to be essential for metabolic activation, DNA adduct formation and liver tumour initiation in infant mice.

In addition, the metabolic formation of N-hydroxylamines and their subsequent oxidation to nitroso derivatives attracts new interest, since uncoupling of mitochondrial respiration by nitrosofluorene and the interference with the mitochondrial permeability transition have been associated with the chronic toxic effects and promoting properties of AAF in rat liver (Klöhn and Neumann 1997; Klöhn et al. 1995; Neumann et al. 1996). No doubt, the contribution of these two major activation pathways depends mainly on the balance between acetylation and deacetylation, and may have important consequences for species-, individual- and tissue-specific tumour formation by aromatic amines. Karreth and Lenk (1991) observed a dynamic equilibrium between N-deacetylation of acetylaminobiphenyl and N-acetylation of 4-aminobiphenyl when measuring directly the appearance of the corresponding metabolites in rat blood. In the present study we demonstrate for the first time the influence of that equilibrium using a biochemical effect marker by directly comparing starting compounds in a different acetylation state.

N-Acetylation of arylamines is catalysed by cytosolic arylamine N-acetyltransferase (NAT) coded for by two gene loci, NAT1 and NAT2. The latter is responsible in humans for the acetylator polymorphism resulting in rapid, intermediate and slow acetylator phenotypes (Grant et al. 1990). 2-AF and benzidine are acetylated by both NAT1 and NAT2 (Grant et al. 1992). However, a single enzyme is able to perform all three types of acetylation, i.e. N-acetylation of arylamines, O-acetylation of arylhydroxylamines and N,O-acetyltransfer with arylhydroxamic acids is catalysed by a single enzyme in rat liver (Land et al. 1993).

Much less is known about deacetylation. Two enzymes have been isolated, which can be inhibited by esterase inhibitors (Jarvinen et al. 1971) and catalyse the hydrolysis of 2-AAF and other amides. The rate of hydrolysis depends on the aromatic moiety as well as on the position of the substituent. A nonspecific carboxyesterase inhibitable by paraoxon has been characterised in rabbit lung and liver, which deacetylates 2-AAF (Aune et al. 1985). At times when research focused on the role of hydroxamic acids, their N,O-transacetylation and the deacetylation of hydroxamic acid esters were emphasised, but little was said about the enzymes involved. Membrane-bound deacetylase(s) or cytosolic N,O-acetyltransferase were considered to activate

N-hydroxy-AAF. Inhibition of mutagenicity by paraoxon indicated a role for deacetylation (Thorgeirsson et al. 1993).

The effects of the acetylator polymorphism on Hb-adducts as a biochemical effect marker further support the role of acetylation and deacetylation for the biological activity of these chemicals. Levy and Weber (1989) have tried to identify the role of N-acetyltransferase activity by producing acetylator congenic mouse lines. These authors found with 2-AF twice as many DNA-adducts in livers of rapid acetylators (C57BL/6J) as in slow acetylators (A/J). This difference increased to 5–7 times the DNA damage in congenic rapid acetylators with slow background (A.B6-Nat^r) compared with slow acetylators with rapid background (B6.A-Nat^s). The formation of DNA-adducts in bladder tissue (Levy and Weber 1992) was later analysed and in contrast higher adduct levels found in slow acetylators. The adduct, however, was in both cases the deacetylated C8 guanine derivative.

Deacetylation activity also underlies genetic variability. Microsomal preparations from the rapid acetylator strain C57BL/6J deacetylate AAF and N-hydroxy-AAF almost twice as efficiently as those from the slow acetylator strain A/J (Hultin and Weber 1987). Rapid acetylators may therefore also be rapid deacetylators. The present results (Table 2) clearly indicate that the extra-hepatic bioavailability of the N-hydroxylamines is greater in the slow acetylating than in the rapid acetylating mouse strain. This finding conforms with the DNA-adduct formation in bladder tissue, but seems to be in contrast to the higher DNA-adduct levels formed in mononuclear leukocytes when these are incubated in vitro with 2-AF (Levy et al. 1994). The true balance between the competing pathways, however, can only be assessed in vivo and it would be interesting to see how these mouse strains differ in their susceptibility to tumour formation.

2-AF was also administered to hamsters of a rapid and a slow acetylator genotype. N-(Deoxyguanosin-8-yl)-2-aminofluorene was the sole DNA-adduct in liver and urinary bladder. The adduct levels were comparable in liver, 4 times lower in bladder tissue, but in this tissue significantly higher with rapid acetylators (Flammang et al. 1992). 2-Aminofluorene Hb-adducts were also measured in this experiment and the levels were higher in slow acetylators.

In humans higher levels of arylamine Hb-adducts in phenotypically slow acetylating individuals have also been observed. Biomonitoring of workers exposed to aniline revealed tenfold higher Hb-adduct levels in slow acetylators (Lewalter and Korallus, 1985). 4-Aminobiphenyl Hb-adducts were present in increasing amounts in nonsmokers, smokers of blond tobacco and black tobacco. In all groups adduct levels were higher in slow than in rapid acetylators (Bryant et al. 1988). Following up bladder tumour patients formerly exposed to benzidine at the workplace, the patients still have the highest 4-aminobiphenyl Hb adduct levels compared to individuals without tumours. Of these 82% were slow acetylators (J. Lewalter, personal communication), which correlates well with the known susceptibility of slow acetylators to occupational bladder tumour formation.

The present experiments primarily demonstrate that a dynamic equilibrium exists between N-acetylation of arylamines and deacetylation of the corresponding acetamides, of which the state in vivo influences considerably the biologically active concentration of critical metabolites as assessed from macromolecular binding. Rather than speaking of N-acetylation as a detoxification step, it may be more appropriate to consider the balance between the two metabolic pathways as indicating a more or less hazardous situation. The results support the use of arylamine Hb-adducts as a surrogate biochemical effect marker to control exposure to genotoxic arylamines.

The relationship between Hb-binding and DNA damage is strongly supported by the linear dose-response relationships shown for binding of AAF to liver DNA as well as to haemoglobin in mice and rats (Pereira et al. 1981), and for binding of trans-4-dimethylaminostilbene to liver and kidney DNA as well as to haemoglobin in rats (Neumann 1984). Yet, neither from DNA damage nor from Hb-binding can risk be calculated readily. Quite a number of additional parameters, chronic toxicity in particular (Neumann et al. 1996), determine location, latency period and incidence of tumour formation. Acetylation and deacetylation, however, are important variables in arylamine metabolism and may influence to a great extent an individuals susceptibility under certain exposure conditions. In this sense, biomonitoring of Hb-adducts may also help to assess relative risks at the level of biochemical effects.

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft. The expert technical assistance of Elke Maurer is gratefully acknowledged.

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